Part One
Upstream Technologies

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Strategies for Plasmid DNA Production in *Escherichia coli*

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1.1
Introduction

DNA-based therapeutics has become an interesting and highly efficient solution for vaccination since its introduction in the 1990s by Wolff *et al.* (e.g., [1, 2]). Since then DNA vaccines have become a viable option to boost the host's immune response for the treatment of bacterial and viral diseases (such as AIDS/HIV, Ebola, and malaria), as well as for the treatment of cancer [3, 4] and even for gene therapy [5]. Principally, DNA can be delivered by viral (generally adenovirus related) or nonviral vector systems. The latter systems include the use of synthetic vectors and the direct application of plasmid DNA. The advantages and disadvantages of each system were recently summarized by Wagner [6].

In a recent very comprehensive review, Kutzler and Weiner summarized the history and state-of-the-art in DNA vaccines. In 2008, four DNA vaccines were approved for veterinary applications and almost 100 clinical trials from phase I to III for human application were underway [7]. Previously, the first DNA vaccine for human therapy was approved in 2003 in China for head and neck squamous cell carcinoma [8, 9]. The numerous clinical trials for plasmid DNA products have demonstrated the safety of the DNA vaccination method and indicate the potential of this relatively new field of therapeutics [10, 11].

The US Food and Drug Administration (FDA) has defined vaccines as [12]:

> . . . purified plasmid preparations containing one or more DNA sequences capable of inducing and/or promoting an immune response against a pathogen. Typically these plasmids possess DNA sequences necessary for selection and replication in bacteria. In addition they contain eukaryotic promoters and enhancers as well as transcription termination/polyadenylation sequences to promote gene expression in vaccine recipients, and may contain immunomodulatory elements.

The present chapter focuses on the production of plasmids for the implementation as DNA-based therapeutics. Process development for efficient production of

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transfection-grade plasmid DNA (i.e., high-quality plasmid DNA), applicable to and functional in cells, tissues, animals, and humans, has raised increasing interest. From the application viewpoint, this is mainly due to the large amounts of DNA vaccine (milligrams) needed for one dose compared to the relatively low amounts (micrograms) needed in the case of vaccination with protein-based antigens [13, 14]). Also, plasmid-based vaccines evoke a weaker immune response compared to viral vectors [15]. Furthermore, plasmid DNA was shown to be much less potent in magnitude and response rate than a viral vector when containing similar antigens. It is assumed that just a very small part of the applied plasmid reaches the nucleus and is expressed [16].

The great advantage of plasmid DNA vaccines is the potential for creating them and establishing their production very quickly, and thus allowing an immediate response to the occurrence of pandemic diseases (e.g., an influenza pandemic) [17, 18]. DNA vaccines can generally be produced within a very short time of 2–4 weeks [17, 19, 20]. In contrast, traditional virus-based vaccines (e.g., an influenza vaccine) require approximately 6 months [17, 21]–a period of time similar to a pandemic [22].

Currently, production of plasmid DNA is exclusively performed in the Gram-negative bacterium *Escherichia coli*. By reviewing the literature, it is remarkable to note that all the general technologies for the plasmid production process itself were basically developed up until and including the 1990s. This includes most of the strains used, the standard techniques for plasmid enrichment in cells (i.e., plasmid amplification), the basics of the high cell density production process, and the initial steps of plasmid purification, generally relying on alkaline lysis [23].

All the cellular factors relating to the production host, the cellular mechanisms for DNA production, and the quality parameters for the DNA product are known in great detail (reviewed excellently in recent papers, e.g., [9, 19, 20, 24]). Nevertheless, practically all of the processes are relatively uniform (i.e., restricted to very few host strains and fermentation procedures). In our opinion, this leaves great room for further process optimization.

1.2 Requirements for a Plasmid DNA Production Process

DNA vaccines consist of plasmid DNA currently exclusively produced in *E. coli*. This Gram-negative bacterium is well known and has been traditionally applied since the construction of the first DNA vectors. The most used strains all belong to the *E. coli* K-12 strains that have been approved by the FDA in different processes. These strains can be applied for recombinant protein and plasmid DNA production.

All *E. coli* K-12 strains originate from a patient stool isolate in 1922, which was applied in the early biochemical genetics studies by E.L. Tatum during the 1940s. Most importantly, these *E. coli* K-12 strains have been subjected to a series of mutagenesis procedures by X-rays, UV irradiation, and nitrogen mustard, which together with selective pressures, spontaneous random mutations, and

chromosomal rearrangements, accumulated mutations that have only been partially characterized [25]. In the 1950s, it was already shown that *E. coli* K-12 strains do not express K and O antigens. Later, they were also shown to be ineffective to infect the human gut. These characteristics made them favorable for laboratory and industrial applications [25].

A number of K-12 strains contain beneficial characteristics for plasmid DNA production, such as mutations avoiding recombination of DNA and stabilizing external DNA. Nevertheless, it was obvious that these strains contain further mutations as a result of the mutagenesis that are unfavorable for industrial production. Only very recently have studies been performed aiming at a better understanding of these relationships by introducing the favorable characteristics into other strains [4], by complementation studies for repair of defective genes [26], and even by genome- or proteome-wide analyses that give a more comprehensive picture [27, 28]. Those studies showed that the traditional strains also include features for an improved synthesis of nucleotides. The higher flux into the nucleotide pathway explains the higher plasmid yield of plasmids. However, the most used strains also contain nonbeneficial mutations and thus the final plasmid yields are not very different from what can be obtained with other strains [4].

Such considerations are becoming more important these days, as competitive efficient large-scale processes are of interest. Generally, the yield of plasmid is relatively low, compared to bioprocesses for protein-based pharmaceuticals or small molecules where volumetric yields are of the order of 10–100 g/l. The yields in plasmid production processes are far below 10 g/l. This low yield contributes partly to the relatively high production costs aside from the costs in downstream purification. The concentration of plasmid DNA per cell weight is usually below 3% dry cell weight [29]. So far, the maximum reported plasmid yield known to the authors is 5% [20]. Thus, in efficient processes with 50–80 g/l of dry cell weight, the volumetric yield would be of the order of 2–4 g/l. The poor growth characteristics of the typical plasmid production strains make even these low yields a challenge, despite the common use of amplification strategies. This underlines the importance of an improved understanding of the genetic factors that influence the plasmid yield in the production process.

Process development is further challenged by the requirements of plasmid quality. Transfection efficiency is highly dependent on the degree of superhelicity. Only densely packed and highly supercoiled plasmids are effectively taken up by the cells [30, 31]. The FDA considers open-circle, nicked, and linear plasmids to be therapeutically less effective in transfection and heterologous expression than supercoiled plasmid DNA. The FDA even states that forms other than supercoiled plasmids have to be regarded as impurities. Consequently, their concentrations must be kept as low as possible in the final products [12].

Finally, there are other requirements for the quality of the plasmid product concerning its purity. Transfection-grade DNA must be endotoxin-free like other therapeutic products from *E. coli*, which sets high demands on the DNA purification process. Although many producers of chromatographic columns in the plasmid purification area offer efficient materials, it is obvious that the acceptance criteria for

plasmid DNA as a drug is challenging. It also gives fresh impetus to applied research in the area of chromatography for a further decrease of the production costs.

1.3 Structure of a DNA Vaccine Production Process

Although DNA vaccines are very popular, only a few recent publications critically review the whole process from molecular biology features through to the bioproduction process and also including the downstream processing [9, 17–19, 29]. Despite this, there exist a large number of excellent and comprehensive reviews on parts of the process, such as on vector design and strain traits [20], state of the fermentation performance and control [32], and the downstream process [9, 13], which are recommended to the interested reader.

The whole process of plasmid DNA vaccine production can be divided into separate steps. Although these steps can be distinguished, they interact in terms of the quality and quantity of the final product. An optimization in one step might have negative effects for the next step.

The different steps in the whole process for a DNA vaccine are:

- The *choice of antigen* affects directly the success of the immune response or gene therapy.
- The *vector construct*, including the choice of replication origin and copy number controlling elements, mainly affects the transcription and translation efficiency. Additionally, the transformation efficiency is affected by the size of the vector. Some structures in the backbone can negatively affect the product quality and yield. The construct also contains the selection system for plasmid maintenance and possibilities for its enrichment.
- The *host strain* produces the plasmid, and has a significant influence on yield and quality. Less important, but also worth considering, is the influence of the strain on downstream processing.
- A well-maintained *cell bank* makes up just a small part of the process, but is fundamental for the quality of the host strain and plasmid.
- The *cultivation medium* has a great influence on growth, metabolism, and consequently plasmid DNA production. In addition, the cell wall structure is also influenced by the growth status of the cell, which affects the cell disruption process and further downstream processing.
- The *choice of process* is a very basic decision for plasmid yield. It depends mainly on the plasmid backbone and the host strain metabolism. Mostly, this is a fed-batch; however, the additives change from case to case.
- The *cell lysis/extraction of plasmid DNA* is, next to the choice of process, a very critical step. At this step, the intracellular plasmid DNA is released by disruption of the cell wall.

- The *clarification of lysate* is the first step in the removal of impurities. The solid fraction with cell debris and precipitations is separated from the soluble fraction, which includes the plasmid DNA.
- *Purification* of the soluble fraction after cell lysis is usually performed by chromatography. This step is a very expensive part due to the use of chromatography columns and solvents as well as the limited capacity of chromatography columns. This step is a major determinant for the quality and purity of the plasmid DNA and the recovery yield.
- With the *formulation*, the purified plasmid DNA is prepared for medical application.

The number of different steps for plasmid DNA production gives us an idea about its complexity. In the following, the basics of each step are described, together with the interactions with other steps.

1.4 Choice of Antigen

The antigen for a DNA vaccine should be selected very carefully, since it provokes the production of the protecting antibodies. It is also part of the plasmid and thus affects the quality of the vaccine.

The main issue for a plasmid DNA vaccine is its effectiveness. With defining a "consensus immunogen" for serotypes or amino acid variation of a pathogen [33], antibodies against a broader set of antigens are produced. Generally, homologies to the host (human) genome may not exist to prevent recombination as a safety issue. Similar to that, Ribeiro *et al.* [34] reported direct repeats to be mutational hotspots, especially in stationary cells. Additionally, genes with the start sequence ATGG are more highly expressed [20], improving the quality of vaccination.

Another very important issue is the codon use of eukaryotic hosts. In many cases, several codons encode for the same amino acid. The preference for one codon over another encoding for the same amino acid is called "codon use," which is specific for different organisms. Since prokaryotes differ in many relevant aspects from eukaryotic cells, it is apparent that the codon encoding the desired protein should be optimized to the host cell. The optimization generates enhanced T-cell response [35, 36] and antibody induction [37, 38].

An important topic is the improvement of immunogenic responses. This can be achieved by cosubmission of plasmids with, for example, interleukin-18 [39] or by application of the DNA vaccine followed by a boost with the modified vaccinia virus Akara platform [40, 41].

For further reading, the interested reader is referred to Kutzler and Weiner [7] and Williams *et al.* [20].

1.5 Vector DNA Construct

Generally, the backbone of the plasmid vector should be as small as possible, since small vectors are supposed to be more potent in transfection than large vectors [42]. Furthermore, the plasmid backbone should carry elements that guarantee a high copy number per cell at the end of the plasmid production process.

1.5.1 Popular Amplification Systems

Mostly, the vector backbone for a vaccine DNA plasmid contains a temperature-inducible pUC origin [43]. The pUC plasmids are derived from plasmid pBR322 and thus contain a ColE1-type origin of replication [44]. The copy number of ColE1-type plasmids is controlled at the level of the interaction of the replication primer RNA II with a small antisense RNA, RNA I. The complex of the two RNAs inhibits the processing of the RNA II primer by RNase H and thus controls the initiation of replication. Additional control is obtained by the small plasmid-encoded Rom protein [45], which stabilizes the complex between RNA II and RNA I (extensively reviewed in [46]).

The *rom* gene is deleted in the pUC vectors, resulting in a higher copy number. Additionally, these plasmids contain an extra point mutation in the RNA II primer, also resulting in a higher basic copy number [47]. Whereas the copy number increase of pUC vectors compared to pBR322 is marginal at 30 °C (approximately 20–30 copies per genome), the copy number is higher at 37 °C (50–70 copies per genome) and even increases further at 42 °C (approximately 130 copies per genome).

A high plasmid copy number may be disadvantageous by provoking a metabolic burden to the host cell that may result in slow growth and vector instability (reviewed in [48]). Thus, the control of the plasmid copy number during the plasmid production process provides a clear advantage. A number of plasmid amplification strategies had already been developed in the 1970s and 1980s.

A historically widely applied method for plasmid amplification was the addition of chloramphenicol to an exponentially growing culture of *E. coli* in concentrations of 10–170 µg/ml [23, 49–53]. This method was even included in the popular guide on molecular cloning by Sambrook *et al.* [54]. The addition of chloramphenicol causes inhibition of the peptidyltransferase and thus inhibits translation. This is connected to an immediate stop of the initiation of chromosomal DNA replication since this depends on *de novo* protein synthesis. However, plasmid replication can still continue for hours. This results in an up to 10-fold enrichment of the plasmid copy number per cell [50, 55]. Reinikainen *et al.* applied this method for plasmid production in a bioreactor, although the final volumetric plasmid yields still remained low due to the use of batch cultivations with LB only [56]. However, although this method is widely applied for laboratory production of plasmids, the

use of the antibiotic chloramphenicol is clearly a drawback for pharmaceutical DNA vaccine production.

A suitable method for pharmaceutical plasmid amplification is the use of temperature-sensitive mutations. These can be located in promoter elements or genes that regulate the initiation of plasmid replication [57–59]. This system can lead to a "runaway" replication with more than 2000 plasmid copies per cell and collapse of cellular functions [60, 61]. Such superamplification systems seem to have their restrictions. As the superhelicity of the plasmid DNA is a major factor in terms of its quality, the maintenance of the energetic status of the bacterial cell is an important factor in process optimization.

Thus, somewhat more moderate approaches like the amplification of *rom*$^-$ plasmids after a temperature shift to a maximum of 42 °C as described by Riethdorf *et al.* [62] have become standard in plasmid production (see also [20]).

A third mechanism for plasmid amplification was described originally by Hecker *et al.* [63]. The authors showed that ColE1-related plasmids are amplified in *E. coli relA* mutants after induced amino acid starvation or amino acid exhaustion. This mechanism was applied for plasmid production in a fermentation process with an *E. coli relA* mutant by control of the amino acid supply [64].

The mechanism of the amplification of plasmids in *E. coli relA* strains was finally resolved by the groups of Wegrzyn and Wang [65]. After it became clear that the different regulation of RNA I or RNA II by direct stringent control is not the regulating mechanism, it was hypothesized that the amplification in *E. coli relA* strains after amino acid starvation is related to a direct interaction of uncharged tRNA species with either RNA I or RNA II [66–68]. This hypothesis also explained the observed differences in plasmid yield after starvation for different amino acids. Recent results of Wang *et al.* indicate that at least in certain cases (as shown for the $tRNA^{Ala}$(UGC)) such a control is not a simple competitive binding, but that ribozyme cleavage activities may be included. The authors could show that alanine starvation in *E. coli relA* mutants leads to RNA I fragmentation at RNA I loop sites that are homologous with the 3′-terminal sequence of $tRNA^{Ala}$(UGC) [65]. Furthermore, they could activate this cleavage and plasmid amplification by overexpression of the $tRNA^{Ala}$(UGC). Although this only proves such a catalytic mechanism in one example, it provokes the hypothesis that this has a wider relevance [69]. Such regulation would directly suggest applications for process development for plasmid production.

1.5.2 Intrinsic Factors

Aside from the specific control of replication, other DNA sequence-related factors influencing plasmid yield, expression, and/or transgene expression in the target organism should also be considered. Williams *et al.* [43] extensively reviewed the current literature and listed "plasmid-intrinsic factors" that reduce the plasmid yield. In one case, the dual terminator sequence upstream of the SV40 enhancer reduced the plasmid yield. The presence of the SV40 enhancer in a pUC-type

plasmid with a cytomegalovirus (CMV) promoter resulted in higher plasmid yields. Also, prokaryotic sequences may provide a direct negative effect on gene expression in eukaryotic cells or may bind to eukaryotic transcription factors (reviewed by [20]).

Strong promoters from human oncogenic viruses were used primarily in early studies, like those of the Rous sarcoma virus [70] or SV40 [71]. Today, promoters of noncarcinogenic sources with similar effectiveness as the CMV promoter [72] are preferred due to safety concerns. Furthermore, the CMV promoter is advantageous by providing a higher constitutive expression level compared to the SV40 promoter [20].

For transcriptional termination of the cloned gene a poly(A) signal site 11–30 nucleotides downstream from AAUAAA (a conserved sequence) is used, which is also required for translocation of the mRNA from the nucleus into the cytoplasm. The bovine growth hormone terminator sequence is widely used [73].

When the gene is transcribed into its mRNA, further obstacles can reduce the effectiveness of the vaccine-like secondary RNA structures and cryptic sequences, which inhibit the export of mRNA [74, 75]. Palindrome sequences and direct or inverted repeats should be avoided, since they represent locations of instability [19], similar to oligopyrimidine or oligopurine sequences [76]. Furthermore, in high copy number plasmids a close or parallel location of the CMV promoter to the replication origin seems to cause replication intermediates, which are fragments of incomplete replication [19].

Williams *et al*. [20] recommend the use of a Kozak consensus sequence [77] to increase expression by the presence of an intron, typically located downstream of the promoter. The interested reader is also referred to this excellent review for an extensive discussion of further elements that affect the efficiency of the construct.

A major concern in plasmid production processes is plasmid stability, which is influenced by the origin of replication (copy number and segregation mode), strain, and cultivation conditions. Host cells that inherit fewer plasmids grow faster, due to the metabolic burden on the host cell caused by the replication of the plasmid and the expression of its genes. Thus, a selective pressure must be provided to prevent a segregational loss of the plasmid and to maintain a high copy number. This is usually achieved with the application of an antibiotic to the growth medium, while the plasmid encodes for a resistance. However, this strategy disagrees with the safety concerns of pharmaceutical products.

Most common is selection with auxotrophies. Here, the genome of the plasmid production host strain lacks an essential gene, mostly for amino acid synthesis, and the same gene is encoded as a transgene on the plasmid. Loss of plasmid induces growth reduction or cell death. Since a small size of the plasmid is important, auxotrophies in tRNA genes, which are encoded by less than 100 bp, fit the requirement well.

As mentioned above, *relA* mutant strains show differences in the starvation response for different amino acids. Wegrzyn [68] showed that starvation of some amino acids results in reduced plasmid replication for different origins. Thus, the

success of plasmid production with a *relA* mutant depends on the constellation of the starved amino acid and plasmid origin.

Aside from antibiotics and complementation of auxotrophic markers, further plasmid selection systems have been developed fitting the claims of pharmaceutical processes. One option is efficient plasmid stabilization systems from plasmids that are tightly controlled by their copy numbers, such as the *parB* locus of the R1 plasmid [78, 79]. This locus is controlled by the expression of two major genes, *hok* (host killing) and *sok* (suppression of killing) antisense RNA. The Sok RNA suppresses translation of the Hok protein, but due to its low stability it is dependent on steady synthesis. Thus, improper plasmid segregation leads to cell killing by the Hok protein. Although R1 is a low copy number plasmid the principle has been shown to also stabilize medium copy number plasmids [80]. Such plasmid stabilization loci rely on the existence of the corresponding genes on the plasmid, which may be a drawback to the use of these systems for therapeutic DNA. This was the driving force for even smaller DNA sequences that are needed for stabilization. This challenge is solved by the operator titrator systems. These systems include very short operator sequences on the plasmid that are not linked to a gene. These operator sequences bind regulatory molecules that otherwise would repress the expression of an essential chromosomal gene. One example of such an operator titrator system has been patented by Sherratt *et al.* [81]. The system works by the control of the expression of an essential chromosomal gene by an operator element that can bind a repressor. The repressor is encoded by a gene that is localized on the chromosome and normally expressed in a low copy number, which is, however, sufficient to repress the essential gene and thus suppress the growth of the strain. Transformation of a plasmid that contains the operator box competes for the repressor *in trans*, and thus the essential gene is derepressed and the cell can grow. This system has been shown to function for a kanamycin resistance gene in connection with the *lac* repressor [82] and also for *dapD* [83], encoding for an enzyme in the diaminopimelate and lysine biosynthesis pathway. Similar systems are applied for different genes (e.g. [83, 84]). In a further system a poison gene was placed in the host genome, while an antidote gene was placed on the plasmid [85]. Cells without plasmid die. A comprehensive review about patents in this area of plasmid production has been published by Carnes and Williams [19].

1.6 Host Strains

The choice of the host strain is a key factor for plasmid yield and quality. Generally, *E. coli* K-12 strains are used for pharmaceutical production as they are "generally regarded as safe" (GRAS) by the FDA. The strains DH5, DH5α, DH1, JM108, and DH10β have been used for efficient transformation and production of plasmid DNA in laboratories for a long time. The preference of these strains for plasmid production [43] has evolved rather due to historical reasons than for

their performance. In relation to large-scale plasmid production it is obvious, and has been discussed in a number of papers, that these strains are not ideal candidates – all of them depend on complex additives in the cultivation medium, and are sensitive to starvation and small changes in the production line. Thus, in most cases the high productivity of these strains in shake flasks is barely transferable to fermentation processes [19, 20]. Generally, all the mentioned strains are very similar in view of their mutations, with the exception of DH10β. Typical beneficial mutations seem to be *recA*, *endA*, *relA*, *gyrA*, and *deoR*, as reported by Jung *et al*. [26].

1.6.1 *endA* and *recA*

Two mutations, *endA* and *recA*, are generally accepted to be important for a high plasmid yield and quality. The *endA1* mutation eliminates the activity of the nonspecific endonuclease I that degrades double-stranded DNA and thus affects the plasmid stability [86]. The gene product of *recA* mediates recombination and thus multimerization of plasmid DNA. The knockout of this function prevents unwanted recombination events [87] and thus stabilizes the size of the plasmid vectors. As plasmid concatemers are avoided, segregational plasmid stability is also increased. *recA* mutations can be found in a great variety of commercial strains designed for plasmid production or cloning purposes. Since pUC-derived vectors do not contain their own multimer resolution site sequence, they require control of multimerization by a *recA* background [20].

Whereas these mutations are clearly necessary, the benefit of the other mutations of the regularly used *E. coli* strains is not so obvious and they may even be disadvantageous for a robust plasmid production process.

1.6.2 *relA*

Most common *E. coli* hosts for plasmid production also contain the *relA1* mutation (Table 1.1). The *relA* gene encodes for the RelA protein, which synthesizes the cellular alarmone guanosine-3′,5′-diphosphate (guanosine tetraphosphate, ppGpp). This highly phosphorylated nucleotide is the regulator of the so-called stringent response, which is induced by amino acid starvation. *relA* mutants with an additional auxotrophic marker in one of the amino acid synthesis pathways are interesting in terms of plasmid production. These mutants are unable to produce the corresponding amino acid, which must be supplemented to the culture medium. As describe above, this can be exploited for plasmid amplification, because plasmid replication continues when the cellular growth stops due to exhaustion or starvation for the respective amino acid. Through the individual control of chromosomal and plasmid replication events, an accumulation of the plasmid by a factor of 6–10 can be provoked. The *relA1* mutation is very common for commercial strains favored for plasmid production, even if in most cases no starvation for a specified

Table 1.1 Selection of strains applied in plasmid production in the laboratory, research, and industry (modified from [88]).

DH5α	F⁻ *Φ80lacZΔM15* Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*($r_k^- m_k^+$) *phoA glnV44 thi-1 gyrA96*(Nal^r) *relA1 spoT* λ^-	*E. coli* K-12, derived from DH1, *hsdR17* inactivates *deoR*
DH1	F⁻ *recA1 endA1 hsdR17*($r_k^- m_k^+$) *phoA glnV44 thi-1 gyrA96*(Nal^r) *relA1* λ^-	*E. coli* K-12, parent of DH5α [89]
JM108	F⁻ *recA1 endA1 gyrA96 thi-1 hsdR17*($r_k^- m_k^+$) *glnV44 relA1* Δ*(lac-proAB)* λ^-	
JM107	*endA1 relA1 gyrA96 thi-1 hsdR17*($r_k^- m_k^+$) *glnV44* Δ*(lac-proAB)* λ^- (F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	*recA*$^+$ variant of JM108, contains F′ plasmid
JM109	*recA1 endA1 relA1 gyrA96 thi-1 hsdR17*($r_k^- m_k^+$) *glnV44* Δ*(lac-proAB)* λ^- (F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	Derived from JM108, but contains F′ plasmid
DH10β	*F⁻ mcrA* Δ*(mrr-hsdRMS-mcrBC) Φ80lacZΔM15* Δ*lacX74 recA1 endA1 araD139* Δ*(ara leu)7697 galU galK rpsL nupG relA1 spoT* λ^-	*E. coli* K-12, MC1061 derivative, K-12 methylase-negative [27]
XL1-blue	*recA1 endA1 relA1 gyrA96 thi-1 hsdR17*($r_k^- m_k^+$) *lac glnV44* (F′ *traD36 proAB*$^+$ *lacI*^{q} Δ[*lacZ*]M15)	Stratagene
SC1	DH5α with *purB*$^+$	[26]
BL21 *endA recA*	*dcm ompT hsdS*($r_B^- m_B^+$)	*E. coli* B [4]

amino acid is specifically applied. The work by Wrobel and Wegrzyn [67] and later by Wang *et al.* [65] elucidated a possible explanation for the amplification of ColE1 plasmids and its interlinkage with amino acid starvation. Their model of the possibility that noncharged tRNAs may interact with the preprimer of replication RNA II or the antisense RNA, RNA I, provides an explanation of why starvation of different amino acids results in different yields of plasmid (i.e., levels of amplification). Thus, for a robust process design it seems absolutely necessary to control carefully the state and kind of amino acid starvation.

Despite these opportunities, from the literature it seems that in current processes the aspect of applying the *relA*-dependent plasmid amplification procedure is not consciously exploited. Also, to our knowledge, no studies have so far been performed to investigate the supercoiling state of such amplified plasmids. It is uncertain whether the high requirements for plasmid quality can be fulfilled by this amplification procedure.

E. coli relA strains often show prolonged lag phases and increased cell death during starvation [90, 91]. Therefore, it is not surprising that strains like DH5α

are usually cultivated on complex or semidefined media. However, in contrast to DH10β with a leucine auxotrophy (*leuABCD*) [27], DH5α is able to grow in mineral salt medium, although with a slow growth rate (doubling time 2–4 h). In this context it is worth mentioning that *E. coli* DH5α is mutated in the *argF* gene (ornithine carbamoyl transferase, arginine metabolism, ornithine cycle), although this mutation does not influence the growth of this strain, as *E. coli* has a functional isozyme, encoded by *argI* [92]. DH5α also has a point mutation in *purB*, encoding for adenylosuccinate lyase, an enzyme involved in nucleoside synthesis. This mutation is responsible for the slow growth of this strain on glucose-based mineral salt medium as reported by Jung *et al.* [26] by complementing DH5α with a genomic library of *E. coli*. The authors succeeded in reverting the strain for faster growth simply by a repair of the point mutation or, alternatively, by overexpression of a functional *purB* gene.

1.6.3 Nucleoside Pathway

Recently, Xia *et al.* [28] published a comparative proteomic study where they documented a higher expression of genes related to nucleoside synthesis in the *E. coli* strains DH5α and XL1-blue compared to W3110, which is generally regarded as a K-12 prototype strain. In their study, three proteins involved in purine nucleotides biosynthesis (PurD, PurC, and PurH) were 2.4- to 5.2-fold upregulated and two enzymes from the glycine synthesis pathway (SerC and GlyA) that is connected to the synthesis of the precursor 10-formyl-tetrahydrofolate. In addition, three enzymes (Cdd, Add, and Udp) involved in the salvage pathway of nucleosides and nucleotides were downregulated. All this may possibly explain the higher yields of plasmid DNA in these strains that are widely applied for cloning purposes. Furthermore, these strains show a higher expression of ribose transporters, which also may be beneficial for nucleoside production.

In earlier studies it was also suggested that the higher transformation rate of DH5α is due to mutation in the *deoR* gene [93]. DeoR is a repressor of the *deoCABD* operon, encoding nucleoside-modifying enzymes, and also represses expression of *nucG*, a nucleoside transporter. Such a mutation would also have clear consequences for the nucleoside synthesis pathway. Nevertheless, in a recent study Xia *et al.* [28] could prove that DH5α does not carry this mutation and their proteomic results showed that the genes that are negatively controlled by *deoR* are not expressed higher in DH5α compared to the wild-type W3110. It is interesting in this context that *E. coli* DH10β does not have the *deoR* mutation, but instead even a mutation in the *nupG* gene, which would be derepressed in a *deoR* background [27].

In our opinion it is very interesting to gain a better understanding of the fluxes into the nucleoside production pathway and the impact on the final plasmid levels. There are few promising examples. Flores *et al.* [94] coexpressed the gene of the glucose-6-phosphate dehydrogenase *zwf*, resulting in a higher growth rate and enhanced plasmid production. Carnes *et al.* [95] have connected *zwf* expression

to the temperature upshift by controlling its expression by the λ temperature-sensitive repressor to support the plasmid production phase.

1.6.4 *gyrA*

Finally, *E. coli* DH5α and a number of similar strains (Table 1.1) contain a mutation in the *gyrA* gene. This gene encodes for subunit 1 of the type II DNA topoisomerase. This enzyme controls the superhelicity of DNA, and is important for healthy DNA replication and distribution to daughter cells. Although this mutation has been mentioned as a positive factor in DH5α [26], we found no further detailed studies in the literature proving a beneficial effect of this mutation. In contrast, for us it seems likely that this mutation could contribute to the poor growth characteristics of DH5α. We believe that our view is supported by the study by Phue *et al.* [4] discussed below, which allowed very high plasmid production in *E. coli* BL21 supplied with only the *endA* and *recA* mutations.

1.6.5 Strains for Production Processes

Understanding the contribution of the mutations in *E. coli* DH5α is very important for the development of processes for DNA vaccines. Although DH5α is considered as a distinguished plasmid producer and is probably the most used strain for plasmid production in the laboratory, it is difficult to cultivate this strain on glucose-based mineral salt medium to high cell densities. Most processes for plasmid therapeutics include complex media components, such as yeast extract or casamino acids. This is unfavorable and different to the state-of-the-art in therapeutic recombinant protein production processes. For recombinant protein production, cultivation on mineral salt medium is preferred generally, as such processes are easier to control than processes with complex additives. The use of mineral salt media is clearly advantageous from the point of process certification and robustness, due to lot-to-lot variations in complex additives, especially of the composition of yeast extract [96].

You *et al.* [88] performed a comparative production of different plasmids in a large number of *E. coli* strains. They suggest that typical protein production strains, such as W3110, TG1, MG1655, and BL21, should not be much worse than the current plasmid production strains, as long as they are supplied with the *endA* and *recA* mutations. According to the authors, the only disadvantage of TG1 is the F′ conjugative plasmid, which may principally provoke conjugative transfer of genetic material to other organisms and thus is unfavorable from a regulatory perspective [20]. Also, it is obvious that the strain BL21 should be applied without the DE3 lambdoid phage, as the DE3 function does not provide any benefit to plasmid production.

Generally, the data from You *et al.* [88] provide a perspective for DNA vaccine process development. Nevertheless, this study should be considered with care, as the results were obtained in shake-flask experiments. As already mentioned above,

it is obvious from history that such data have a low relevance for the development of high cell density cultivation processes.

Real evidence for this hypothesis from a real bioproduction approach was recently published by Phue *et al.* [4], who confirmed that BL21 supplied with the *endA* and *recA* mutations is a superior host compared to DH5α. The authors obtained a volumetric plasmid yield of 2 g/l for BL21 *endA relA* and only half of it from the same process with DH5α. This may be considered as a breakthrough paper, as it paves the way for more straightforward process development of plasmid-targeting processes. Considering these results it seems that the host strain can be freely selected, and can be subsequently supplied with the *endA* and *relA* knockouts by widely used chromosomal knockout methods as described, for example, by Datsenko and Wanner [97]. These knockout strains may be complemented by further advantageous factors. For instance, Ow *et al.* [98] have recently shown that a knockout of *fruR* encoding for the global regulator Cra can further increase the plasmid yield.

In our opinion all these results are difficult to understand, as extensive metabolic flux analyses for the involved pathways have not yet been published. It is assumed that such future analyses will contribute to even higher yields and more stable processes.

The creation of new production strains by the methods discussed above would also have direct consequences for the preservation of strains. Although various standard methods for preserving *E. coli* in cell banks have been well established over the years [99–101], strains with certain genomic mutations usually recover less efficiently from cryopreservates than wild-type cells without such mutations. This has been studied especially with *E. coli relA* strains – the allele that is carried by most of the strains generally applied for plasmid production (see above). These strains show a longer lag phase at the beginning of cultivation and only recover slowly from a nutritional stress ([91] and our own unpublished results). This is likely a result from a prolonged use of reserves after a nutrient stress if the stringent response is not activated. Furthermore, *relA* mutants respond differently to cold shock [102].

The slower recovery of relaxed cells from nutritional changes has also been explained by Lrp, which is necessary for the fast adaptation of the cell to changes in the nutritional conditions [90]. *E. coli relA* strains have lower Lrp levels and thus exhibit longer lag phases after a nutritional change. Thus, it can be concluded that *E. coli relA* mutants are more sensitive and should be handled with care after stress, whereas wild-type cells are more robust and easier to handle.

1.7 Cultivation Medium and Process Conditions

The cultivation medium has a major impact on growth and plasmid yield [32, 103]. In optimal processes, plasmid yields of 1.5 to over 2 g/l of supercoiled plasmid are obtained.

In most studies of plasmid production, complex media or semidefined media with yeast extract and/or casamino acids are applied. This is a drawback (for a good review on the advantages of defined media, see [104]) as:

- Achieving high cell densities with complex media is generally challenging and the metabolism is not easily controlled. Thus, such processes provide usually lower cell densities and show a lower robustness. These effects are caused by the high metabolic fluxes and their changes when shifting to higher cell densities. Nevertheless, high cell densities are the basis for efficient plasmid production. Also, a major problem of complex additives is the lot-to-lot variation in the composition of the components, especially in the case of yeast extract [96].
- Processes based on chemical ingredients of nonanimal origin have distinct advantages in view of Good Manufacturing Practice production and for FDA approval. Thus, new approaches for the selection of plasmid-producing strains that can be grown on standard phosphate-buffered high cell density media is an important and long neglected aspect.
- Mineral salt media also have considerable advantages with regard to the scale-up of bioprocesses. While inactivation of components is a major issue for complex media, mineral salt media are robust when considering sterilization [105, 106].

Only a few studies have investigated the impact of the composition of a complex or semidefined media on plasmid production in detail. One example is the cultivation of the pUC-derived plasmid pSVβ in DH5α in a semidefined medium with casamino acid addition by O'Kennedy *et al.* [103]. The authors found a significant influence of the carbon/nitrogen ratio of the medium on the plasmid DNA yield per cell. The yield varied within one order of magnitude with the best result at a carbon/nitrogen ratio of 2.78 : 1. Furthermore, the extracted DNA showed less contamination with chromosomal DNA. It is questionable if the same can be found for a fully defined medium and for another strain–plasmid constellation. Nevertheless, it is worth further investigation.

Generally, in batch studies for plasmid production, glycerol has been applied as the most common carbon source, as glycerol provokes low acetate production compared to glucose. The advantage of using glycerol as a carbon source is related to the fact that in most cases a temperature upshift is applied to boost plasmid replication. If glucose is applied the temperature upshift would provoke a high amount of acetate formation during the plasmid amplification phase, which is detrimental to the process [4]. Alternatively, it is a standard procedure to avoid acetate production by a glucose-limited fed-batch. However, importantly in such a process the feeding conditions should also be adapted after a temperature upshift to avoid glucose accumulation and acetate overflow metabolism. Considering this, it is important to note that acetate-based overflow metabolism is much higher in *E. coli* K-12 strains compared to *E. coli* B strains [107], which makes the latter excellent for production also with glucose as a carbon source. Phue *et al.* [4] considered this when they introduced the *endA* and *recA* mutations into a BL21 background and obtained a plasmid yield of 2 g/l with cultivation on complex

medium. However, as this strain can also be grown on mineral salt medium, it would be interesting to see what the plasmid yields would be in a typical glucose- or glycerol-limited high cell density fed-batch process.

Typical carbon source-limited fed-batch processes can be performed to very high cell densities with *E. coli*, which may exceed 100 g/l cell dry weight [108]. They are normally performed in a two-step process. A fed-batch process typically starts with a batch phase, where the carbon source is unlimited. This is followed by a fed-batch phase where the carbon source is added in controlled limiting amounts. Due to the fast metabolism of *E. coli* the concentration of the carbon substrate in the bioreactor is close to zero. The specific growth rate in the feeding phase is generally far below the maximum specific growth rate so that aerobic conditions can be maintained despite the limited oxygen transfer rate. Thus, in most fed-batch processes the specific growth rate is kept relatively low, in the range of 0.05–0.2 h^{-1}. The low growth rate should be favorable for the plasmid copy number [109, 110]. Generally, high cell density processes can be easily performed with mineral salt media without any complex additives, but are more difficult to control when complex additives are needed.

Variations on this basic standard procedure can be produced to combine such a fed-batch process with plasmid amplification. Plasmid amplification is provoked by different changes according to the origin–host constellation. A prominent example is the temperature-sensitive pUC plasmids, which are able to amplify to very high copy numbers after an increase of the cultivation temperature to 42 °C. With this procedure Williams *et al.* [43] obtained a yield of 2.22 g/l plasmid, corresponding to 5% of dry cell weight, in a medium-density fed-batch process with DH5α. This process applies a kind of standard procedure with batch and fed-batch phases. The authors apply an exponential feed rate that supports a growth rate of 0.12 h^{-1}. Before induction of plasmid amplification the culture is grown at 30 °C and a temperature shift is performed later to 42 °C. The plasmid production phase lasts for 5 h (see also [111]). In this phase it is important for a high plasmid yield to either apply optimized fermentation media [112] or to additionally control the fluxes towards the synthesis of nucleosides like in the process by Carnes *et al.* [95].

In some processes the production phase is followed by a further "hold" phase [43]. In the process by Williams *et al.* for the cultivation of DH5α with a plasmid containing a pUC-derived origin, after amplification at 42 °C the cells were held for 0.5 to more than 3 h at 25 °C before the fermenter content was cooled down to 15 °C for harvest. This "hold" step increased the plasmid quality and yield. Goldstein and Drlica [113] found that the plasmid linking number increases with decreasing temperature and that it takes about 2 h after a shift from 37 to 15 °C to adjust this linking number. A change in linking number shows a shift in the band pattern of isolated plasmid on chloroquine/agarose gel electrophoresis, which indicates changed physicochemical properties. These properties might be important for purification or therapeutic effectiveness in gene therapy [114].

Other alternative strategies for plasmid amplification at the fed-batch fermentation scale that do not rely on a temperature upshift are the addition of

chloramphenicol [56], which is not preferred due to high costs and safety concerns, and amino acid starvation [64] (for discussion, see Section 1.6). However, it seems that both of these processes have not hitherto been carried further to production scale.

1.8 Lysis/Extraction of Plasmid DNA

The plasmid produced by the host strain must be released to the medium. This can be achieved by disruption of the cells. Since plasmids are susceptible to physical stress, cell disruption by ultrasound or bead mills is not preferred. Generally, mechanical disruption is not favorable due to shear damage, which might produce chromosomal DNA fragments of similar behavior at purification as the desired plasmid DNA [115].

The two most common lysis procedures for the extraction of plasmid DNA are alkaline lysis [23] and heat lysis. Both are applicable to large-scale production, but with restrictions.

Heat lysis requires an elevated temperature. This takes a long time for a large-scale cultivation vessel. Carnes and Williams [19] report a patent [116] where the harvested biomass is suspended in STET buffer and pumped through a heat exchanger. An optional use of lysozyme increases the plasmid concentration by 4–5 times. On the contrary, Watson [117] indicates that the majority of costs for heat lysis are accounted for by lysozyme. The lysate is centrifuged down and the formation of open-circle plasmids could be reduced with 100 mM EDTA. A higher plasmid recovery was reported with heat lysis than by chemical lysis.

The alkaline lysis provides a disadvantage in large-scale vessels, too. The lysis is performed best at a defined pH. pH gradients occur by the addition of highly alkaline solution to the suspension due to insufficient mixing at a large scale and thus the efficiency of the lysis procedure may be affected. Additionally, it was shown that the lysis step itself takes just up to 40 s for DH5α [118]. Free plasmids exposed to high pH (pH 13) are known to denature irreversibly [17, 19, 23]. In addition, the neutralization might occur after more than the referred time due to insufficient mixing. Hoare *et al.* [17] suggest the addition of neutralizing potassium acetate stream to be as cold as possible. This application would increase the burden of handling a proper alkaline lysis even more. The impact of lysis time, pH, and mixing was evaluated by Meacle *et al.* [119].

A pipe system, where the reagents are mixed for the optimal time or the fermentation broth is heated, provides a solution for both kinds of large-scale lysis procedures. A corresponding solution has been performed by Carnes *et al.* [19] who use static mixers for alkaline lysis. Many of alkaline lysis technologies are licensed (summarized in table 2 in Carnes *et al.* [19]).

Autolysis of the bacterial cells would have a huge process advantage in terms of alkaline lysis and heat lysis, since both need further equipment for proper handling at a large scale. Induced autolysis, however, needs no further equipment and

can be performed in the cultivation vessel. Furthermore, no expensive reagents (lysozymes for heat lysis) are necessary or dilution and potential denaturation of the plasmid DNA (alkaline lysis) occur. The major disadvantage is the release of chromosomal DNA, which is not denatured during lysis, contrary to alkaline and heat lysis. A solution to this problem might be the coexpression of an exonuclease. It is also very likely that chromosomal DNA is retained in the cell when the cell wall is not broken entirely, but contains pores though which the plasmids can leave the cells.

Carnes and Williams [19] also mention patents for autolysis. In one system [120] the lysozyme remains in the cytoplasm until holin is coexpressed, which forms a channel to the periplasm. In another patent [121] the endolysin gene from bacteriophage λ is induced by arabinose. Carnes *et al.* [95] describe an autolysis strategy where the engineered strain expresses a heat-inducible endolysin that is coexpressed during the plasmid amplification phase. After harvest the cells were treated with STET buffer, and the released plasmid fraction was very pure and only contained a low concentration of genomic DNA. In other cases, lysis of the cells is performed by a simple freeze–thaw cycle.

After autolysis, the chromosomal DNA is usually not denatured, but fragments of chromosomal DNA can negatively influence the final plasmid yield. Nevertheless, Carnes and Williams [19] suggest autolysis to have a tremendous potential since the lysis step is a bottleneck in alkaline and heat lysis. As a solution for the high chromosomal DNA content they suggest the use of exonucleases like bacteriophage T5 exonuclease, which digests linear single- and double-stranded DNA [122], but leaves supercoiled plasmid DNA intact. A corresponding high yield process has been established [19, 123].

1.9 Purification

Plasmid purification processes involve cell disruption prior to the intermediate and final purification steps. There exist different methods to isolate the plasmid DNA after its release from the cytoplasm, like methods aiming at selectively precipitating the plasmid DNA or precipitating impurities (as intermediate purification steps). In addition, filtration techniques, aqueous two-phase systems, and reverse micellar phase extraction can also be applied as intermediate purification steps. Some of these approaches are more useful for the laboratory scale; others are also suitable for large-scale applications. Chromatographic techniques are mostly employed for final purification leading to the desired purity.

The starting point for the purification of plasmids is the lysate with a complex composition containing the plasmids in a diluted form. Although in most lysis procedures a large portion of chromosomal DNA is eliminated, there are still significant amounts of soluble contaminants present in the lysate, like host proteins, endotoxins (lipopolysaccharides), and structurally related impurities such as RNA and remaining chromosomal DNA.

Some quality criteria for plasmid DNA are listed by Carnes and Williams [19]:

Proteins	undetectable (<0.5%)
RNA	undetectable (<1%)
Chromosomal DNA	undetectable (<1%)
Endotoxins	<10 EU/mg plasmid
Plasmid isoforms	<5%
Percentage of covalently closed plasmid DNA	>90%

For clinical applications, it is generally preferable to avoid the use of RNase, which is typically of bovine origin, due to the possibility of contamination with bovine spongiform encephalitis agent. Bacterial products for pharmaceutical use are better treated under conditions free of animal-derived products. Therefore, recombinant bovine pancreatic RNase-expressing *E. coli* strains have been developed [124, 125]. The RNase accumulates in the periplasmic space and mixes with RNA at lysis. Nevertheless, it is overall favored not to add enzymes (like proteinase K or RNase) to plasmid preparations, because the enzymes have to be removed afterwards. Toxic agents (e.g., CsCl, CsBr, phenol) must be avoided. Naturally, they are not certified for application in humans.

1.9.1 Clarification of the Lysate and Intermediate Purification

The purification process starts with a clarification of the lysate, whereby the cell debris, host proteins, and undesired nucleic acids are removed. This is done by precipitation, centrifugation techniques, and filtration.

Precipitation of impurities can be applied as intermediate purification, for instance prior to chromatography. For example, the removal of RNA is important, because it reduces the capacity in anion-exchange chromatography [126]. A precipitation of RNA can be achieved by addition of calcium chloride in addition to poly(ethylene glycol) (PEG) and NaCl, avoiding the use of RNase [127]. The cationic detergent cetyltrimethylammonium bromide (CTAB) can be applied to remove proteins, RNA, endotoxins, and chromosomal DNA [29]. Antichaotropic salts (such as ammonium sulfate, tripotassium acetate, ammonium acetate, and calcium chloride) are suitable to remove proteins, endotoxins, and higher-molecular-weight RNA, but also chromosomal DNA (calcium chloride) [29, 128–130]. A method for selective RNA removal is metal-affinity precipitation. This approach is based on the affinity of divalent transition metal ions to aromatic nitrogens due to π–d orbital overlap [131]. Balan *et al.* [132] describe the binding of RNA with chelated copper and its subsequent removal by coprecipitation with a copolymer of *N*-isopropylacrylamide. Nevertheless, this method may not be suitable for large-scale processes because of low performance.

In addition to precipitation of impurities, there exist different approaches for selective precipitation of plasmid DNA. With different concentrations of the cationic detergent CTAB, plasmid DNA can be partially purified from clarified STET lysate (used for heat autolysis) [133, 134], whereby precipitations of the

different compounds occur within a narrow concentration range of CTAB. Although the CTAB concentrations can be fine-tuned in a way even allowing a separation of topological isoforms of plasmids like with chromatographic approaches, a drawback in scaling up CTAB precipitation is the concentration distribution and control in a large tank [29]. Therefore, it may not be a suitable alternative for chromatographic techniques in large-scale bioprocesses. Further precipitation agents are PEG [135, 136], as well as high salt concentrations [135].

As a further possibility to capture plasmid DNA, Wahlund *et al.* [137] describe the utilization of the polycation poly(*N*,*N*′-dimethyldiallylammonium) chloride for selective precipitation. The method can be applied also at higher concentrations of potassium acetate present in a lysate prepared by alkaline lysis. This results in a reduced sample pretreatment. The effectiveness of subsequent chromatography steps may be increased. Based on triple-helix interactions, a method is described by Costioli *et al.* [138] that combines precipitation with the specificity of affinity. Triple-helix interactions are based upon the formation of a triple-helical structure, whereby a single-stranded polypyrimidine oligonucleotide interacts with a corresponding polypuridine sequence in a double-stranded DNA target molecule [139]. This interaction is reversible. In the method of Costioli *et al.* [138], the target DNA is bound by an oligonucleotide linked to a thermoresponsive *N*-isopropylacrylamide. By a temperature shift, the whole complex can be precipitated. However, limitations for triple-helix precipitations are slow binding kinetics [140], hampering application for large pharmaceutical processes.

Lao *et al.* [141] describe a further method for affinity precipitation of plasmid DNA, whereby a specific DNA-binding protein binds the target DNA. The DNA-binding protein undergoes a reversible, temperature-triggered phase transition, so that the plasmid DNA is precipitated by a temperature shift. This protocol is suitable for the laboratory scale. Generally, added agents must be certified for application in humans when using them in pharmaceutical processes according to the FDA or European Medicines Agency guidelines.

Like precipitation, filtration can be applied as an intermediate purification step prior to chromatography, as described by Guerrero-Germán *et al.* [142], but it can also be used in some cases as a final polishing step. Filtration is suitable for pharmaceutical processes on a large scale if, of course, agents not certified for application in humans are avoided. Tangential flow filtration (TFF) is capable of separating plasmid DNA and RNA because of their size difference. Plasmid DNA remains in the retentate, whereas RNA flows through and accumulates in the permeate. Eon-Duval *et al.* [128] describe a process combining TFF with a calcium chloride precipitation step resulting in complete removal of RNA and high recovery of plasmids. Other papers [143–145] also describe the use of TFF. TFF is applicable at a larger scale. Filtration techniques are furthermore suitable for sterilization, as shown by Watson *et al.* [146].

Apart from selective precipitation, different authors describe selective adsorption of impurities like chromosomal DNA, open-circular plasmid, and endotoxins by crystalline calcium silicate hydrate and its derivatives [147, 148]. Further possibilities for intermediate purification are aqueous two-phase systems (ATPSs) and

reverse micellar phase extraction. ATPSs for plasmid purification are mainly polymer/salt systems like PEG/potassium phosphate [149–151] and polymer/polymer systems like PEG/dextran [152] and thermoseparation EO-PO/dextran [153, 154]. In these systems, the plasmid accumulates in the most suitable environment. This system can be applied right after lysis, as in WO2004020629 [155], where the plasmid in the top phase is enriched after heating. Unwanted plasmid forms may result from damage, possible at any stage [115, 156]. ATPSs serve as approaches for the initial purification of plasmid DNA from the main impurities, which has been described by several authors [149, 150, 154]. The ATPS in combination with a membrane step has been shown to be an alternative, chromatography-free process for purification of plasmid DNA, which is also promising for application at large scales [157]. Streitner *et al.* [126] published a process employing reverse micellar phase extraction to remove especially RNA from a lysate, which is then suitable for a subsequent anion-exchange chromatography step.

1.9.2 Purification by Chromatography

Purification of plasmid DNA is most widely done by chromatographic methods. However, chromatography systems were developed and optimized for the separation of smaller molecules. Chromatography procedures of plasmid DNA on conventional particle-based stationary phases are slow, diffusion-controlled processes, leading to time-consuming separations. Carnes and Williams [19] describe the downstream process of plasmid DNA as the most cost-intensive part of a plasmid DNA production process. Methods that offer good performance are often not suitable for large scale because of high costs and a low binding capacity [19]. Low capacities arise from an inaccessible inner surface of particle-based stationary phases. Owing to the large size of plasmids, they tend to adsorb mostly at the outer surfaces [158, 159].

Even porous particles seem to have too small pores to allow large plasmid molecules to diffuse into them [160]. To the authors' knowledge, there exists a very promising alternative – monolithic stationary phases with very large pores, resulting in easily accessible binding surfaces and convective flow. Chromatography on porous monolithic stationary phases, which will be described Section 1.9.2.5, is suitable for laboratory-scale plasmid purification as well as for large-scale applications. For completeness, some chromatography techniques applying conventional particle-based stationary phases will be illustrated very briefly in the following, whereby anion-exchange chromatography may be the most important mode. Sousa *et al.* [9] give a detailed overview of chromatographic plasmid purification methods.

1.9.2.1 Anion-Exchange Chromatography

The phosphates of the nucleic acid backbone charge DNA negatively, so it can interact with positively charged groups of anion-exchange chromatography

stationary phases. At suitable salt concentrations, the plasmid DNA binds to the anion-exchange resin, while genomic DNA and RNA flow through the column. With an optimized elution gradient, a separation of different topological isoforms of plasmid DNA is also possible. Details of anion-exchange chromatography are given in the literature [161–167]. Improvements in the binding capacities and throughput of anion-exchange chromatography have been made. Some experimental evidence showed that a compaction of plasmid DNA has a positive effect on chromatographic separations [130]. It has been shown that a compaction of nucleic acids results in an enhanced binding capacity in anion-exchange chromatography [168].

1.9.2.2 **Hydrophobic Interaction Chromatography**

In this type of chromatography, the differences in surface hydrophobicity of supercoiled plasmid DNA, RNA, endotoxins, single-stranded DNA, relaxed double-stranded DNA, and proteins are used for separation by salt-promoted binding, whereby ammonium sulfate is widely used. This is based on the displacement of ordered water molecules from the regions around the surfaces of the molecules, thus more nonpolar regions on the molecule surface are presented that can bind to a hydrophobic stationary phase. Elution is done by a decrease of the salt concentration [151, 161, 169–171]. Diogo *et al.* [172] describe a scalable purification process for plasmid DNA on hydrophobic interaction chromatography Sepharose™.

1.9.2.3 **Gel Filtration**

Purification of plasmid DNA by gel-filtration/size-exclusion chromatography has been described by several authors, also partly at the preparative scale [130, 161, 173]. Some improvements were obtained by varying water-structuring salts and their concentrations in the running buffer (NaCl and $(NH_4)_2SO_4$) leading to compaction of plasmid DNA [174, 175], which is shown to be beneficial for the binding capacity [168].

Other modes of interaction for chromatographic purification of plasmid DNA have been applied to a lesser extent. Different examples of affinity modes can be found, such as thiophilic interaction chromatography [176, 177], triple-helix interaction [178, 179], and immobilized metal ion affinity chromatography (IMAC) [180]. Due to metal ion interactions with aromatic base nitrogens, IMAC stationary phases are capable of selectively binding single-stranded nucleic acids. However, the similar properties of plasmid DNA and chromosomal DNA limit purification with IMAC [181]. Further strategies are protein–DNA interaction, as published by Darby *et al.* [140], in combination with IMAC. Other approaches for protein/peptide–DNA chromatography have also been described [182–184]. An amino acid–DNA affinity chromatography mode with histidine and arginine promoting specific interaction with plasmid DNA has also been published [185, 186].

1.9.2.4 **Membrane Chromatography**

Limitations in chromatographic purification of plasmid DNA when applying conventional particle-based stationary phases arise from inaccessibility of the binding

surface and slow diffusion processes. These restrictions can be overcome by using membrane chromatography. Membranes can be seen as very thin beds. Membrane chromatography supports exhibit large pores, resulting in a better accessibility to binding sites and enabling convective flow. They provide a lower pressure drop and allow a higher velocity. The consequence is a higher productivity [187, 188]. Endres *et al.* [187] report a 20–25 times greater dynamic binding capacity and 55–550 times greater flow rate compared to anion-exchange beads. Zhang *et al.* [189] and Yang *et al.* [190] describe scalable processes based on membrane anion exchangers that may be suitable for pharmaceutical processes. Guerrero-German *et al.* [142] describe a tandem process using two anion-exchange membranes consecutively. Nevertheless, some drawbacks of applying membrane chromatography arise from relatively large dead volumes and problems with uniform flow distribution. Stacking of membranes increases the void volume [191].

1.9.2.5 **Chromatography on Porous Monolithic Supports**

As mentioned previously, the use of most conventional particle-based chromatographic materials for the purification of plasmid DNA often results in a low performance, because the large plasmid DNAs tend to adsorb predominantly at the outer surfaces of the beads, which reduces the binding capacity [159, 192]. The use of particle-based stationary phases with larger pores can indeed improve the mass transport, but those materials provide a smaller surface area for the binding of molecules. Plasmid separation with conventional particle-based chromatographic materials is thus made difficult by mass transfer limitations or low binding capacity [193].

It is important to consider the pore characteristics of the chromatographic material when the target molecule has a very large hydrodynamic radius and thus a low molecular diffusion coefficient, as in the case of plasmid DNA. An alternative option in chromatographic purification of plasmid DNA is the use of monoliths (continuous beds) with large pores (up to 5 μm). Those monolithic stationary phases exhibit the advantage of enhanced mass transport due to convectional flow, resulting in a very fast transfer between the mobile and the stationary phase. This is possibly due to the macroporous character, whereby the mobile phase is forced to flow through the large pores. The long diffusion time required when using conventional particle-based chromatographic materials is drastically shortened. A further advantage is a reduced pressure drop [194–197].

The large pores of monoliths provide a good accessibility for plasmid DNA to the internal surface of the material [192] resulting in an enhanced binding capacity, reaching up to 10 mg plasmid DNA/ml chromatographic material [198]. Therefore, monoliths could be the preferred materials for plasmid separations [193].

A further important feature of monoliths is that the resolution and binding capacity is not affected by the velocity [199–201]. This property is an important factor in scale-up for preparative applications (e.g., in the form of a radial chromatography mode) [200, 202, 203].

Moreover, the separation of different topological isoforms of plasmid DNA (and multimeric forms) is possible with monolithic stationary phases. This is important because the covalently closed circular form (supercoiled) is considered as the most

appropriate form, especially for therapeutic applications. According to international regulations, the content of the covalently closed circular form in plasmid preparations has to be above 90% [204, 205]. It is reported that separation with monoliths results in plasmid DNA consisting of up to 98% of the covalently closed circular form [159]. It is also published that all critical elements in a plasmid DNA purification process (detergents, enzymes, and organic solvents) could be avoided when applying a chromatographic approach with monoliths [191].

Short monolithic columns are not only suitable for preparative applications, but also for analytical approaches (e.g., for in-process control of plasmid purity) [191, 206]. Yu *et al.* [191, 207, 208] published a method applying miniaturized monolithic columns based on hydroxyapatite prepared by the sol-gel technique. This method allows the purification of plasmid DNA at a microscale that meets miniaturized and automated trends of genetic analysis and for high-throughput applications.

The monolith technique is commercially available. The advantages mentioned above all serve for high-throughput plasmid DNA purification. When monolith chromatography was established for large-scale production, the calculations showed a drastic decrease in costs per milligram of plasmid. Independent of any innovations in the plasmid DNA vaccine production process, the use of monolith technology is recommended due to its advantages [191, 195, 198, 206, 209].

1.10 Formulation

At formulation, an active drug is prepared and mixed with other compounds so that it can be stored, shipped, and applied. One important aim of formulation is maintenance of the chemical and physical stability to achieve the desired shelf-life. The large plasmid DNA in "naked" form is relatively sensitive to degradation by plasma and serum proteins *in vivo* [210]. Furthermore, it is sensitive to mechanical forces like shear stress [211], which can lead to a conversion of topological isoforms (e.g., supercoiled to open circular form). Plasmid molecules in aqueous solution can be prone to hydrolytic and oxidative changes leading to damage to deoxyribose, the bases, or the phosphodiester backbone. For instance, acidic conditions accelerate the hydrolytic degradation of plasmid DNA; transition metals can cause oxidative damage [156, 212, 213].

Raising the pH and addition of chelators or free radical scavengers are methods used for stabilization [156, 212]. Lyophilization can be also applied in pharmaceutical processes to prolong stability. Shen *et al.* [214] describe a formulation process whereby DNA is lyophilized under maintenance of the product activity. Ingredients used for stabilization of plasmid molecules have to be safe for pharmaceutical applications. Different agents from a GRAS library were tested by Zeng *et al.* [215] regarding their ability to stabilize plasmid DNA. Sodium citrate, malic acid, ethanol, and Pluronic® F-68 were found to be effective stabilizers. Other agents were identified as destabilizing, including ferrous chloride, ascorbic acid, human serum albumin, and PEG 1000.

Apart from chemical and physical stabilization, formulation should ease delivery of the active compound. The right formulation of plasmid DNA can ease delivery and enhance the desired stimulation of the immune system response [216–218]. Generally, plasmid DNA in a condensed form is better protected against chemical degradation as well as against physical damage [219]. Thus, formulation processes very often aim at condensation of plasmid DNA. This condensation process of plasmid DNA is widely done by neutralization of the negatively charged backbone. Therefore, small cationic reagents are utilized that are capable of binding to the phosphate groups of the DNA by means of electrostatic interactions. This leads to a decrease of the volume occupied by the DNA molecule [29].

One challenge for compaction/condensation is that neutralization can cause physical instabilities and interactions of the particles, which can result in aggregation [219–222]. To compact and protect the plasmid DNA, vector systems based on cationic lipids and cationic polymers are used. Encapsulation of the plasmid DNA molecules can furthermore assist targeting to a specific cell type, leading to an enhancement of the delivery efficacy (e.g., by conjugating the vector with specific ligands such as antibodies) [220, 221]. The vectors can be modified towards slow degradability or stimuli reactivity (e.g., pH or temperature sensitivity) [223]. Additional molecules like short-chain polymeric alcohols (e.g., PEG) improve the half-life of the vector in the body [224]. Bolhassani [225] gives an overview of different delivery systems.

1.10.1 Lipoplexes

Plasmid DNA electrostatically interacts with cationic lipids. Those complexes are called lipoplexes. Thereby the cationic lipids serve to condense the DNA via charge interactions [226]. Entrapping plasmid DNA within cationic liposomes can result in greater humoral and cell-mediated immune responses against the encoded antigen compared to similar amounts of the naked plasmid DNA [227, 228]. The enhanced immune response of liposome-entrapped plasmid DNA may be due to facilitating the uptake of the plasmid DNA by antigen-presenting cells [229]. Furthermore, plasmid DNA inside cationic liposomes is reported to be protected from degradation by nucleases [230, 231]. Torchilin [232] gives a general review about liposome-based formulations. Myschik *et al.* [233] reviews various aspects of lipid-based vaccine formulations.

1.10.2 Polyplexes

Plasmid DNA can also form stable complexes with cationic polysaccharide/polymers due to electrostatic interactions. Different polycations have been investigated for gene delivery, such as poly(L-lysine) [222, 234, 235], polyethyleneimine, amine-functionalized polymethacrylates, poly(lactide-*co*-glycolide)s, cationic poly(β-amino)esters, poloxamers, and polyvinylpyrrolidone polymers (reviewed

by Kutzler and Weiner [7]), block copolymers (e.g., poly(L-lysine)-*b*-PEG) [223], and chitosan [236, 237]. Nevertheless, some systems based on polycations show some cytotoxicity or a low efficacy for transfection [238, 239]. Some pentablock copolymers were shown to have good biocompatibility [240].

1.10.3 Inorganic Nanoparticles

A further strategy for delivery is the use of nanoparticles (calcium, magnesium, and manganese phosphates) encapsulating plasmid DNA. The encapsulated DNA is protected against DNases, and was used for transfer *in vitro* and *in vivo*. The surface of those nanoparticles can be as well conjugated with different ligands [241–243].

1.11 Conclusions

Plasmid production for DNA-based therapeutics has become an established technology in the pharmaceutical industry. The technology and its application potential is only at the beginning, especially if the large field of vaccines is considered. In comparison to other biological processes the yields of plasmid on a volumetric basis are relatively low. However, it can be assumed that efforts in the area of the development of new production strains and new amplification techniques in line with a more detailed understanding of the metabolic fluxes towards nucleotide synthesis will provide a basis for improved processes. The final yield and quality of the DNA products will also gain from new developments in the field of downstream processing.

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